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Myofibroblastic conversion of mesothelial cells

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Background. The continuous chemical, physical, and inflammatory insults of prolonged continuous ambulatory peritoneal dialysis (CAPD) incite mesothelial cell responses, which may result in peritoneal fibrosis. The transforming growth factor-β (TGF-β), especially the isoform TGF-β1, has long been known to play crucial role in the fibrogenic process. Although several studies have implicated TGF-β in peritoneal fibrosis, the underlying mechanism has not been completely elucidated.

Methods. To test the effects of exogenous TGF-β1 on mesothelial cells, we assessed cytoarchitectural changes of human peritoneal mesothelial cells (HPMC) in in vitro culture by light, immunofluorescent, electron and immunoelectron microscopy, and differential gene expression analysis using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and cDNA expression array assays.

Results. The TGF-β1–induced myofibroblastic conversion was a transdifferentiation process resulting in characteristic myofibroblastic phenotype that included prominent rough endoplasmic reticuli (rER) with dilated cisternas, conspicuous smooth muscle actin (SMA) myofilaments, frequent intercellular intermediate and gap junctions, and active deposition of extracellular matrix (ECM) and formation of fibronexus. The gene expression array analysis revealed complex modulation of gene expression involving cytoskeletal organization, cell adhesion, ECM production, cell proliferation, innate immunity, cytokine/growth factor signaling, cytoprotection, stress response, and many other essential metabolic processes in mesothelial cells.

Conclusion. This report describes myofibroblastic conversion of mesothelial cells, a previously undefined, yet frequently speculated, cell adaptive or pathogenic process. Our study helps to elucidate the complex molecular and cellular events involved in myofibroblastic conversion of mesothelial cells. We propose that differentiated epithelial cells of mesothelium convert or transdifferentiate into myofibroblasts, which implies the recruitment of fibrogenic cells from mesothelium during serosal inflammation and wound healing.

Key words: myofibroblasts, mesothelial cells, TGF-β1, peritoneum.

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Overproduction of extracellular matrix (ECM), accompanied by the presence of activated myofibroblasts and local expression of transforming growth factor-β (TGF-β) are the typical manifestations of pathologic fibrosis and these have been particularly well demonstrated in kidney, liver, and lung fibrosis [1]. The spatial and temporal orchestration of myofibroblasts in these pathologic processes implies a dynamic nature of this type of cells. Quiescent myofibroblasts exist in many normal tissues or organs as specific stable cells with the potential to be drafted into fibrogenic action. These cells include tissue fibroblasts, various types of smooth muscle cells (SMAs), hepatic stellate cells, renal mesangial cells, intestinal pericryptal cells, pulmonary interstitial contractile cells, pericytes of blood vessels and reticular cells in lymph nodes, to cite the principal cell types [2, 3]. They reside mainly as stromal cells with or without myoid features. In contrast to mesenchymal stromal cells, epithelial cells are infrequently converted into myofibroblasts in mature tissues or organs. Recently, we have observed definite myofibroblast conversion, both functionally and morphologically, from human differentiated mesothelial cells. These findings are clinically relevant because perturbation of mesothelium is unavoidable in many major therapeutic procedures such as continuous ambulatory peritoneal dialysis (CAPD) and abdominal or thoracic surgery. The demonstration that TGF-β stimulates the mesothelial-myofibroblastic conversion further implies the recruitment of fibrogenic cells from mesothelium during serosal inflammation and wound healing.

METHODS

Human peritoneal mesothelial cells culture and TGF-β1 treatment

We cultured human peritoneal mesothelial cells (HPMCs) from healthy mesenteric or omental tissues of consenting patients undergoing elective abdominal surgery. We first selected an intact mesothelial membrane and then firmly clamped it to a base of cylindrical rings of various diameters (2 to 5 cm) to form isolation wells. The HPMCs were detached from the serosa by

trypsin (0.05%, wt/vol) digestion and resuspended in growth medium [Dulbecco's modified Eagle's medium (DMEM)/F-12] (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Life Technologies, Grand Island, NY, USA), antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) (Gibco), and 2 mmol/L L-glutamine. We used a panel of antibodies to check every batch of initially isolated mesothelial cells to ensure they were positive for mesothelial markers (cytokeratin, vimentin, calretinin, and Wilms' tumor 1 protein [WT-1]), whereas negative for endothelial (Ulex Europaeus lectin, type 1 [UEA-1] and factor VIII), smooth muscle (desmin) and fibroblastic specific antibody (ASO2) markers (micrographs not shown). The majority of initial culture exhibited typical cobblestone appearance that represented pure mesothelial cells. The propagated cells were harvested and frozen for further use. In all experiments, we used HPMC from the same pooled stocks of several donors. The confluent HPMCs were transferred to lower serum-containing medium (2% FBS) for 48 hours prior to receipt of treatment. The HPMCs were continuously exposed to various concentrations of recombinant human TGF-β1 (0.3 to 25 ng/mL) (R&D Systems, Minneapolis, MN, USA) for 1 week and then transferred to original culture medium containing 10% FBS for 1 more week. We have prepared control groups that were parallel duplication of experimental groups in terms of cell batch, growth condition, and culture period.

Histology and immunohistochemistry

We grew HPMC on either Transwell membranes (Transwell-Clear 3452; Costar, Cambridge, MA, USA) for ultrastructural and immunoelectron microscopic studies or in slide flasks (Lab-Tek II; Nalge Nunc, Naperville, IL, USA) for immunofluorescent studies. The primary antibodies for immunofluorescent staining of HPMC included those to collagen I (rabbit, 1:20, Novocastra, Newcastle-upon-Tyne, UK), collagen III (goat, 1:20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-smooth muscle actin (α-SMA) (mouse, 1:40, Signet, Cheshire, UK), pan-cytokeratin (mouse, 1:50, Sigma), cytokeratin AE1/AE3 (mouse, 1:40, Sigma), desmin (mouse, 1:40, Sigma), WT-1 (rabbit, 1:50, Santa Cruz), calretinin (rabbit, 1:20, Zymed, San Francisco, CA, USA), and fibroblast-specific ASO2 (mouse, 1:20, Dianova, Hamburg, Denmark). The secondary antibodies were either rhodamine or fluorescein-conjugated antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Nuclear counterstains were either 4,6-diamidino-2-phenylindole HCl (1 μg/mL) or propidium iodide (0.5 μg/mL). We used the pre-embedding immunogold method to demonstrate the distribution of type I collagen in HPMC. The HPMCs growing on Transwell membranes were initially fixed in freshly prepared 3% paraformaldehyde in phosphate buffer and subsequently treated with 0.1% sodium borohydride and 0.05% Triton X-100. After antibody incubation [primary antibody, rabbit anticollagen I, 1:100, Novocastra; secondary antibody, ultrasmall gold-conjugated goat antirabbit immunoglobulin (IgG), $F(ab')_2$, 1:100, Aurion, The Netherlands], HPMC was further processed through postfixation (2.5% glutaraldehyde), silver enhancement (R-Gent SE-EM, Aurion, The Netherlands), and osmication (0.5% OsO_4). The HPMC was then dehydrated and embedded according to routine procedures. The membranes embedded in epoxy resin were sectioned through different directions to facilitate the observation of three-dimensional changes of the cells. Thin sections were viewed and digitally recorded using a transmission electron microscope (JEM-1230, JEOL, Japan) equipped with multiscan CCD camera (791 MSC; Gatan, Pleasanton, CA, USA).

Reverse transcription-polymerase chain reaction and cDNA expression array analyses

For reverse transcription-polymerase chain reaction (RT-PCR) analysis of HPMC, total RNA was extracted with RNAzol B (Tel-test, Inc., Friendswood, TX, USA). Contaminating genomic DNA was removed with RNAase-free DNAase following the manufacturer's protocol. First-strand cDNA was synthesized from total RNA with Superscript II RNase H^- Reverse Transcriptase (Gibco BRL, Life Technologies) using oligo(dT) as primers. Semiquantitative evaluation of collagen I (COL1A1) and α-smooth muscle actin (ACTA2) mRNA transcripts by RT-PCR were performed using following primers: COL1A1 sense 5′-GGCGGCCAGGGCTCCGACCC-3′, antisense 5′-AATTCCTGGTCTGGGGCACC-3′; ACTA2 sense 5′-GCTCACGGAGGCACCCCTGAA-3′, antisense 5′-CTGATAGGACATTGTTAGCAT-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense 5′-CCACCCATGGCAAATTCCATGGCA-3′, antisense 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ was used as an internal control. Template concentration was titrated to yield exponentially amplified products between 17 and 23 cycles (30-second denaturation at 94°C; 30-second annealing at 62°C; and 30-second extension at 72°C) (thermal cycler; Perkin-Elmer 2400, Norwalk, CT, USA).

For cDNA expression array analysis (Atlas™ Human 3.6 Array, Clontech, Palo Alto, CA, USA), we exposed HPMC in two different concentrations of recombinant human TGF-β1 (1.25 and 5 ng/mL) for 1 week. Untreated HPMC was used as control. The cDNA probes were prepared from HPMC according the manufacturer's instruction (Atlas Pure Total RNA Labeling System, Clontech). Briefly, HPMC was lysed and total RNA was isolated by two runs of phenol:chloroform extraction. Total RNA was further processed through isopropanol precipitation, alcohol wash, and DNAse I digestion. Poly

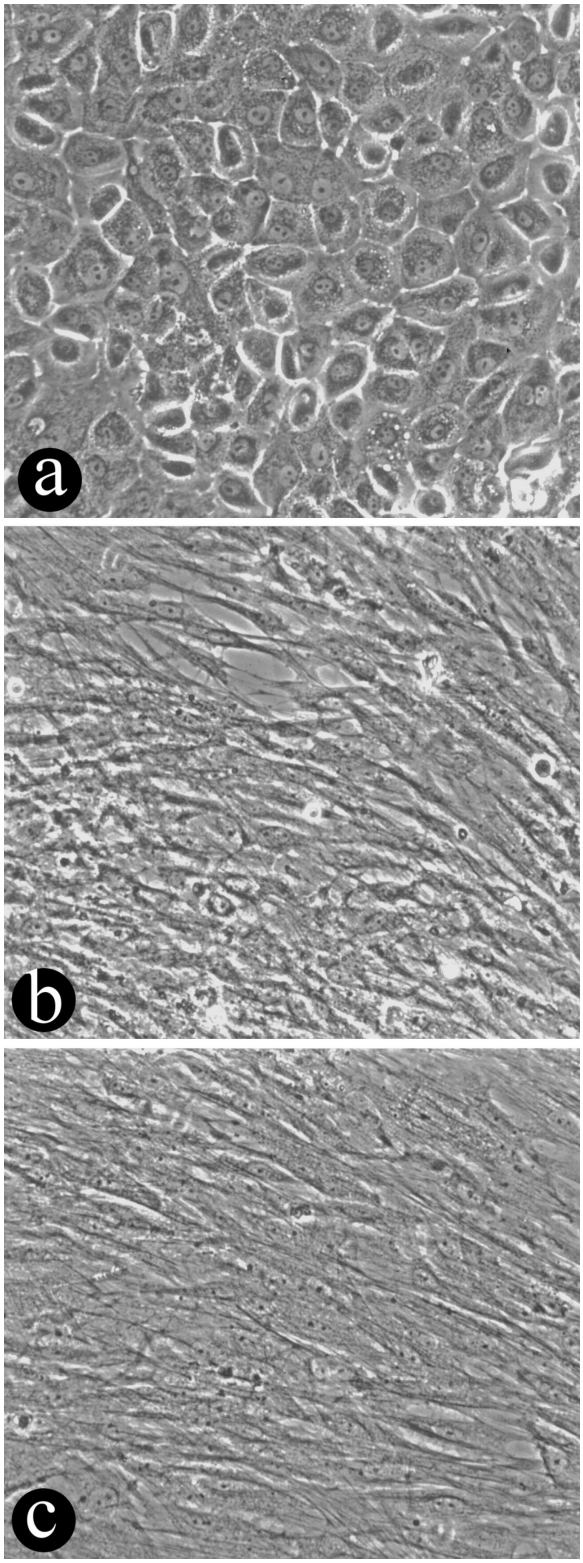

Fig. 1. Phase-contrast microscopy of myofibroblastic conversion. (*a*) The control mesothelial cells without treatment of transforming growth factor-β1 (TGF-β1) exhibited epithelial cell morphology with characteristic cobblestone-like growth pattern. (*b*) After continuous treatment of TGF-β1 (0.6 ng/mL) for 7 days, the epithelial morphology of mesothelial cells completely converted to spindle fibroblast-like morphology with multilayered crisscross growth pattern. (*c*) The mesothelial cells still maintained their myofibroblastic phenotypes after withdrawing TGF-β1 for 7 days.

A+ mRNA was isolated from total RNA using biotinylated oligo(dT) mRNA and streptavidin magnetic beads. cDNA probes were synthesized by Moloney murine leukemia virus (MMLV) reverse transcriptase in the presence of cDNA synthesis (CDS) primer mix and [α-^{33}P]-dATP (>2500 Ci/mmol;) (Amersham, Piscataway, NJ, USA). Labeled cDNA was purified by column chromatography (Nucleospin Extraction Kit, Clonetech). The membranes of cDNA expression arrays were prehybridized with 0.1 mg/mL of sheared salmon testes DNA (Sigma) at 68°C for 30 minutes. cDNA probes were hybridized to the arrays at 68°C overnight. Membranes were washed four times with 2 × saline sodium citrate (SSC) containing 1% sodium dodecyl sulfate (SDS) and twice with 0.1 × SSC (containing 0.5% SDS) for 30 minutes at 68°C. Acquisitions of images were processed by a PhosphorImager (Molecular Dynamics, Amersham). The data were analyzed using a specific software for membrane arrays (AtlasImage 1.5, Clontech). Paired arrays were normalized by averaging the corresponding expression of three housekeeping genes (*ubiquitin*, *GAPDH*, and *cytoplasmic β-actin*). We define significant differential expression of a given gene by greater than 2.0-fold and 25 intensity value (the minimal differential expression level).

RESULTS

TGF-β1 induced morphologic and functional reformation in differentiated human mesothelial cells

To determine the direct response of mesothelial cells to TGF-β1, we incubated isolated HPMC in TGF-β1–containing medium (0.3 to 25 ng/mL). The mesothelial cells started to take on the myofibroblastic phenotype after 72 hours of treatment (Figs. 1 and 2). The minimal dose of TGF-β1 to induce the full spectrum of changes was 0.6 ng/mL. Morphologic features of these converted cells met the stringent definition of the myofibroblast, and these criteria include (*1*) conspicuous bundles of actin micro (myo) filaments with interspersed dense bodies running parallel to the long axis of cells, (*2*) active deposition of ECM, (*3*) well-developed cell-ECM assembly (fibronexus), (*4*) frequent intercellular intermediate and gap junctions, and (*5*) prominent rough endoplasmic reticuli (rER) with dilated cisternas [4–6] (Fig. 2). We demonstrated that myofibroblasts were collagen (type I)-secreting cells (Figs. 2 and 3). Immunoelectron microscopy localized type I collagen mainly to the pericellular matrix and in collagen secretion granules (Fig. 2). Immunofluorescent and semiquantitative RT-PCR studies confirmed that type I collagen and α-SMA were de novo synthesized after TGF-β1 stimulation, while type III collagen contributed little to the ECM synthesis (Fig. 3). The myofibroblastic conversion was accompanied by the expression of the human fibroblast marker ASO2 [7] and by the loss of the calretinin (a calcium-binding protein constantly

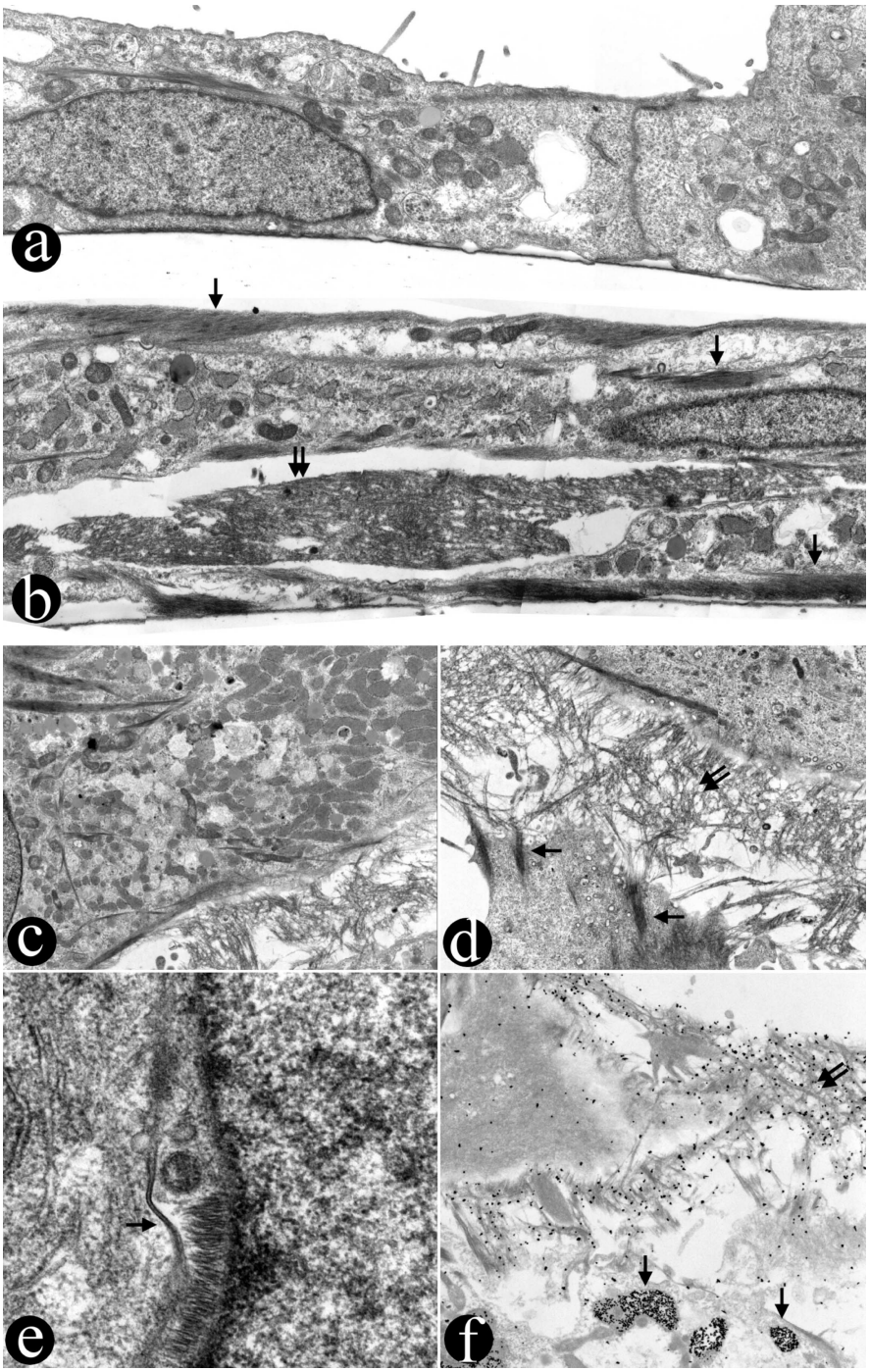

Fig. 2. Electron microscopy of myofibroblastic conversion. (*a*) The control mesothelial cells without treatment of transforming growth factor-β1 (TGF-β1) showed typical epithelial monolayer growth with scattered surface microvillus structures. (*b*) TGF-β1–induced myofibroblastic conversion is characterized by spindle multilayered growth, well-developed actin bundles decorated with dense bodies running through the cytoplasm (arrows), and abundant extracellular matrix formation between cell layers (double arrows). (*c*) Abundant rough endoplasmic reticuli (rER) is well demonstrated in this horizontal section of a myofibroblast. (*d*) Horizontal section of myofibroblasts demonstrated numerous convergence of cytoplasmic myofilaments and extracellular matrix (ECM) fibrils forming a fibronexus (arrows). The ECM was mainly composed of randomly oriented collagenous fibrils (double arrows). (*e*) The junctions between myofibroblasts were principally intermediate or gap junctions (arrow). (*f*) Immunogold stain confirmed the distribution of type I collagen in pericellular matrix (double arrows) and secretion granules (arrows) of the myofibroblasts.

expressed on normal mesothelium) [8] (Fig. 3). The down-regulation of calretinin was further proved by cDNA expression array analysis (Table I, group H). The myofibroblasts still expressed prototypical mesothelial markers, cytokeratin and vimentin (Fig. 3, photomicrograph of vimentin not shown). The preservation of cytokeratin in all culture cells indicated that there was no contamination of fibroblasts. The myofibroblasts phenotype persisted at least 1 week after TGF-β1 was withdrawn (Figs. 1 and 3).

The survey of gene expression associated with myofibroblastic conversion

The further investigation of the expression of 3528 genes (Atlas™ Human 3.6 Array, Clontech Laboratories) revealed that 52 genes altered their expression during myofibroblastic conversion, according to our definition of significant differential expression (≥2.0-fold increase and 25 intensity value) (Table 1). Among these genes, 23 had enhanced expression while 29 showed suppressed expression. For the convenience of discussion,

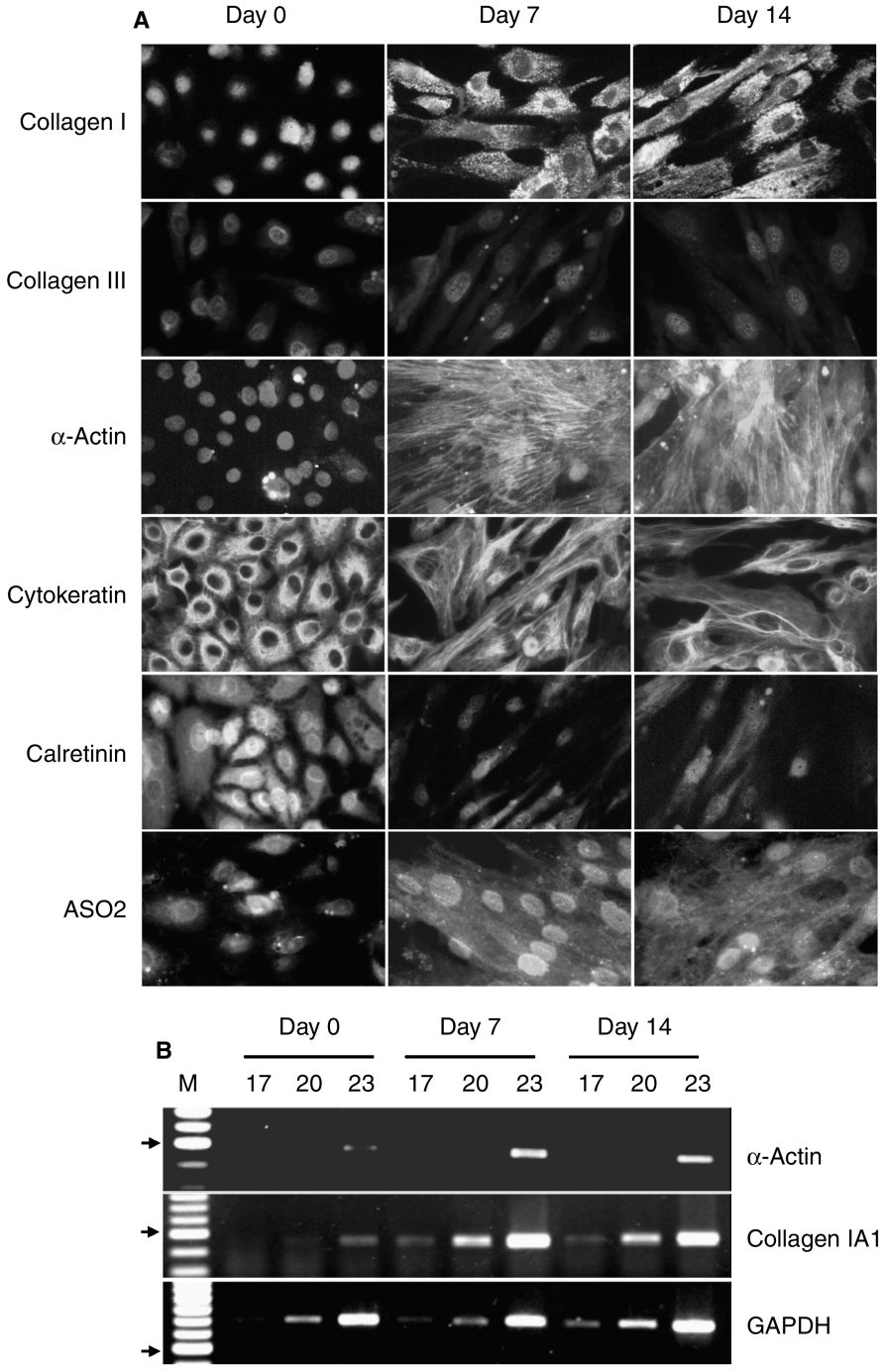

Fig. 3. Immunofluorescent and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) expression profiles in the process of myofibroblastic conversion. (*a*) Immunofluorescent expression profiles of type I collagen, type III collagen, α-smooth muscle actin (α-SMA), cytokeratin, calretinin and ASO2. (*b*) Semiquantitative RT-PCR analysis of mRNA expression of α-SMA and type I collagen. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal control. Day 0, the control mesothelial cells without treatment of transforming growth factor-β1 (TGF-β1); day 7, after continuous treatment of TGF-β1 (0.6 ng/mL); day 14, TGF-β1–treated cells (day 7) maintained another 7 days in TGF-β1–free medium. M, 100 bp DNA ladder (MBI Fermentas, arrows 400 bp); 17, 20, and 23, amplification cycles of PCR.

we categorize these genes into eight groups according to their functional correlation (Table 1, groups A to H). The significance and influence of these gene expressions in myofibroblastic conversion of mesothelial cells are described below.

DISCUSSION

Myofibroblastic phenotype is dynamically modulated and is heterogeneous. It might express some specific morphology or function in a given organ or tissue. For this reason, it is difficult, if not impossible, to determine whether a myofibroblastic differentiation is partial or complete. Despite the inherent heterogeneity of myofibroblast, the essential phenotypical components in an activated prototypical myofibroblast, for example, myofibroblast in granulation tissue, could serve as a baseline of myofibroblastic differentiation. These components, mainly in ultrastructural and immunohistochemical context, would be prominent rER, conspicuous peripheral

Table 1. Differential gene expression associated with myofibroblastic conversion

Symbol	Full name	Genbank	DEL[a]
	Group A		
TPM1	Human skeletal muscle α-tropomyosin	M19715	+454
CD44	CD44 antigen (homing function and Indian blood group system)	M59040	+39
ARHA	Ras homolog gene family, member A	L25080	+32
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	X69550	−50
MLCK	Myosin light chain kinase	U48959	−58
VIL2	Villin 2 (ezrin)	X51521	−107
TMSB4X	Thymosin-β-4, X chromosome	M17733	−2413
	Group B		
CDH2	Cadherin 2, type 1, N-cadherin (neuronal)	M34064	+186
TIMP-1	Tissue inhibitor of metalloproteinase 1	X03124	+164
MMP-11	Matrix metalloproteinase 11 (stromelysin 3)	X57766	+125
SERPINE1	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	X04429	+77
COL4A2	Procollagen IV α 2 subunit	X05562	+49
ITGAV	Integrin, α V (vitronectin receptor, α polypeptide, antigen CD51)	M14648	+32
LOX	Lysyl oxidase	M94054	+25
ITGB8	Integrin, β_8	M73780	−30
CDH5	Cadherin 5, type 2, VE-cadherin (vascular epithelium)	X79981	−63
	Group C		
CCR2	Chemokine (C-C motif) receptor 2	U03882	+25
BST1	Bone marrow stromal cell antigen 1	D21878	−26
C1S	Complement component 1, s subcomponent	X06596	−49
C3	Complement component 3	K02765	−67
BF	B factor, properdin	X72875	−231
	Group D		
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	U09579	+62
PCNA	Proliferating cell nuclear antigen	M15796	−34
PSMC3	Proteasome (prosome, macropain) 26S subunit, ATPase, 3	M34079	−35
DAPK-1	Death-associated protein kinase 1	X76104	−39
JUN	v-jun avian sarcoma virus 17 oncogene homolog	J04111	−146
	Group E		
SKIL	SKI-like	X15219	+74
SKI	v-ski avian sarcoma viral oncogene homolog	X15218	+57
EGR-1	Early growth response 1	M62829	+51
INHBA	Inhibin, β A (activin A, activin AB α polypeptide)	J03634	+41
BMPR1B	Bone morphogenetic protein receptor, type IB	U89326	+28
	Group F		
VEGF	Vascular endothelial growth factor	M32977	+359
IL-6	Interleukin 6 (interferon, β 2)	X04602	+161
SCG2	Secretogranin II (chromogranin C)	M25756	+70
LIF	Leukemia inhibitory factor (cholinergic differentiation factor)	X13967	+52
PDGFRA	Platelet-derived growth factor receptor, α polypeptide	M21574	+38
TNFAIP2	Tumor necrosis factor, α-induced protein 2	M92357	−30
IL-6ST	Interleukin 6 signal transducer (gp 130, oncostatin M receptor)	M57230	−40
	Group G		
ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	U07919	−43
CYP1B1	Cytochrome P450, subfamily I, polypeptide 1	U03688	−61
HSP90A	Heat shock 90 kD protein A (Hsp90A; Hsp86; Hspca)	X07270	−70
HSPA8	Heat shock 70 kD protein 8	Y00371	−73
SEPP1	Selenoprotein P, plasma, 1	Z11793	−142
	Group H		
MAN2B1	Lysosomal α-mannosidase	U60266	+42
SNRPE	Small nuclear ribonucleoprotein polypeptide E	X12466	−26
PTPN11	Protein tyrosine phosphatase, nonreceptor type 11	L08807	−30
CALB2	Calbindin 2, (29 kD, calretinin)	X56667	−39
MFI2	Antigen p97 (melanoma associated), melanotransferrin	M12154	−46
T-STAR	Sam68-like phosphotyrosine protein, T-STAR	AF069681	−55
HNRPU	Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	X65488	−57
ADFP	Adipose differentiation-related protein	X97324	−69
NPC2	Niemann-Pick disease, type C2	X67698	−74

The genes in each category are ranked according to the differential expression levels (DEL).
[a] +, increased expression; −, decreased expression

myofilaments with focal density, fibronexus junctions, a prominent Golgi apparatus producing collagen secretion granules, ECM deposition, gap, and intermediate junctions, and positive for vimentin and α-SMA [2, 4]. Although expansion or reduction of these components in an acceptable range to reflect the consequences of functional modulation would not hamper myofibroblastic designation, it would be favorable to use more stringent criteria in a given experimental system to avoid misinterpretation of nonspecific cellular adaptive changes. In this report, we demonstrate the full-spectrum myofibroblastic conversion of mesothelial cells that exhibits all essential components of a prototypical myofibroblast. In addition, the presence of residual cytokeratin in all our myofibroblasts directly indicate their origin from mesothelial cells and is an unequivocal evidence against contamination of myofibroblast of other origin.

To further understand the internal processes of mesothelial-myofibroblastic conversion, we compared the differences of their gene expression. In the present study, we found altered expression of several genes that regulated, either directly or indirectly, the actin-based cytoskeleton (Table 1, group A), and a second set of genes involved in ECM assembly and cell adhesion (Table 1, group B). The up-regulation of small guanosine triphosphatase (GTPase) Rho, specifically by Rho A (ARHA), and down-regulation of Rho guanosine diphosphate dissociation inhibitor (ARHGDIA) and myosin light chain kinase (MLCK) might contribute to actin-myosin filament assembly, fibronexus formation, and cell contraction during myofibroblastic conversion [9]. One of the ezrin-radixin-moesin (ERM) proteins, ezrin (villin 2, VIL2), and its transmembrane anchorage protein, CD44, were altered during myofibroblastic conversion. Both proteins are essential for Rho-induced cytoskeletal effects, specifically the stress fiber formation [10]. Since ezrin (villin 2) is also a component of epithelial microvilli, its down-regulation might result in loss of mesothelial microvilli during myofibroblastic conversion [11]. The thymosin-β-4 (TMSB4X) is the main intracellular actin–sequestering peptide and serves as an actin buffer [12]. Down-regulation of thymosin-β-4 (TMSB4X) implied the release of actin building blocks for myofibroblastic conversion. Like kidney mesangial myofibroblasts, mesothelial myofibroblasts also expressed abundant sarcomeric thin filament proteins [skeletal muscle α-tropomyosin (TPM1)] [13].

Both cadherins and integrins are also subjected to the regulation of GTPase Rho A [14, 15]. It is worth noting that mesothelial cells specifically expressed N-cadherin (CDH2) and VE-cadherin (CDH5). The up-regulation of N-cadherin and down-regulation of VE-cadherin (Table 1, group B) during myofibroblastic conversion implied that myofibroblasts altered homotypic adhesive properties and might therefore promote their migration and spreading by loosening VE-cadherin–associated adheren junctions and enhancing adhesion with other N-cadherin–expressing cells such as smooth muscle cells and fibroblasts. Given that αv integrin combines with 1 of 5 beta integrins (β_1, β_3, β_5, β_6, or β_8) and recognizes the sequence RGD in a variety of ligands [16], the up-regulation of αv and down-regulation of β_8 implied that cell-ECM interactions were of the mesenchymal rather than epithelial type during myofibroblastic conversion. In addition to type I collagen, other ECM might be produced. The increased production of basement membrane material in myofibroblasts was implied by the enhanced expression of procollagen IV α2 subunit (COL4A2). The matrix degradation might be suppressed in myofibroblasts, since expression of tissue inhibitor of metalloproteinase (TIMP-1) and serine proteinase inhibitor (PAI-1) was enhanced. In addition, the expression of metalloproteinases (MMPs) was not enhanced in myofibroblasts except for expression of MMP-11 (stromelysin 3). The main function of activated MMP-11 is not to hydrolyze major components of ECM but to hydrolyze α1 serine PAI [17].

Myofibroblastic conversion also implies alteration of the innate immune system in serosal cavities (Table 1, group C). Mesothelium is an important barrier against potential pathogenic microorganisms in body cavities. Both C1s and B-factor (BF) are essential for the activation of C3 by generation of C3 convertase through the classical and alternative pathway respectively. The deficiency of complement would lead to ineffective clearance of bacteria on mesothelium [18]. However, the depression of the complement system, especially C3, might reduce bacterial colonization as has been demonstrated on renal tubular epithelium [19], and therefore reduce the bacterial translocation and reservoir in the peritoneum. The human mesothelial cells constitutively express bone marrow stromal cell antigen 1 (BST-1, CD157), a cell-surface glycoprotein that facilitates B lymphopoiesis in bone marrow [20, 21]. Despite that the functional role of mesothelial BST-1 is not clear, the alternation of BST-1 in peritoneum might significantly affect local extralymphoid B-cell differentiation. In addition, low BST-1 might be necessary to maintain fibroblast morphology since CD157-transfected fibroblasts lost typical elongated spindle morphology [22]. Mesothelial cells also constitutively express monocyte chemotactic protein 1 (MCP-1) and its binding receptor (CCR2). The enhanced CCR2 expression in myofibroblasts would probably promote haptotactic migration in response to MCP-1 and play an important role in myofibroblast recruitment during inflammatory reactions [23].

The myofibroblastic conversion might result in quenching cell proliferation through the enhanced expression of cyclin-dependent protein kinase inhibitor, $p21^{cip1}$, and the depression of proliferating cell nuclear antigen (PCNA) (Table 1, group D). $p21^{cip1}$ can interact with PCNA,

blocking its activity necessary for DNA replication [24]. JUN, a major component of the activator protein-1 (AP-1) transcriptional complex, was also down-regulated during myofibroblastic conversion. The suppression of JUN might hinder cell cycle progression because it has been shown that constitutive c-Jun inhibits p21 induction [25]. Recent work indicates that many important regulators of G_1 and S phases are targeted for ubiquitination and subsequent degradation by the 26S proteasome (PSMC3) [26]. The inhibition of proteasome (PSMC3) activity would thus reduce cell proliferation. Our results implied that TGF-β1 is predominantly a cytodifferentiating rather than a proliferating growth factor. Myofibroblastic conversion did not seem to enhance apoptosis since the major mediators of programmed cell death were unaltered except for the down-regulation of death-associated protein kinase-1 (DAPK-1), a positive mediator of apoptosis [27]. Our previous observation that tumor necrosis factor-α (TNF-α), but not TGF-β, activated Fas/Fas-ligand pathway to promote apoptosis of mesothelial cells also supports this assumption (unpublished observation).

The modulation of TGF-β signaling pathway (Table 1, group E) during myofibroblast conversion implied negative feedback regulation with up-regulation of v-ski avian sarcoma viral oncogene homolog (SKI) and SKI-like (SKIL) (SnoN) and concomitant down-regulation of the 26S proteasome (PSMC3). It is speculated that mild-to-moderate feedback up-regulation of these co-repressors is mainly for fine-tuning of the selective suppressive effects of TGF-β and is functionally distinct from their activity in oncogenic transformation, which is usually promoted by highly expressed SKI/SnoN [28, 29]. The down-regulation of cellular proteasomes might reduce the degradation of SnoN and SKI. TGF-β enhanced expression of the inhibin/activin βA subunit (INHBA) gene and might be responsible only for up-regulation of activin A (βA-βA) in that inhibin α as well as activin βB and βC subunits were not expressed during myofibroblast conversion. Our results also suggested that several other factors might enhance TGF-β signaling system. These factors included BMPR1B (ALK6), an alternative type I receptor for TGF-β agonists [30], early growth response-1 (EGR-1), an early response gene whose product directly transactivates TGF-β in an autocrine fashion [31], and leukemia inhibitory factor (LIF), a cytokine of the interleukin-6 (IL-6) family that in conjunction with BMP2 acts through STATs, Smads, and coactivator p300 to activate transcription [31].

The expression profiles of cytokine and growth factor systems implied involvement of vascular endothelial growth factor (VEGF), IL-6, and platelet-derived growth factor (PDGF) in myofibroblast conversion (Table 1, group F). The strong augmentation of VEGF expression was not accompanied by the expression of its three major tyrosine kinase receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR). and VEGFR-3 (Flt-4). It thus implied that TGF-β directs myofibroblasts to mediate paracrine signaling of angiogenesis. This finding has potential clinical significance because recent investigation on VEGF has recognized its pathogenic role in peritoneal microvascular dysfunction and ultrafiltration failure. [32–34] Our finding is further supported by a recent in vivo study that transduction of TGF-β1 gene to peritoneal mesothelial cells not only enhances VEGF production but also induces accumulation of α-SMA–positive interstitial cells in peritoneal membrane. [35] The response of the IL-6 family is similar in that IL-6 and LIF are up-regulated in myofibroblasts. Other factors of IL-6 family were not expressed. The mesothelial cells seemed to be the primary IL-6 unresponsive cells expressing only IL-6 signal transducer (IL-6ST, gp130) and no gp80. The gp130 was further down-regulated after myofibroblast conversion. Thus, the IL-6 produced is speculated to mediate paracrine signaling of other types of cells in the vicinity of active myofibroblasts. This functional property of myofibroblasts has been frequently described in association with tissue repair and the fibrogenic process [36]. The enhanced expression of PDGF receptor α polypeptide gene (*PDGFRA*) without concomitant expression of *PDGFRB* gene implied that αα-receptor homodimer was the predominant PDGF receptor on the myofibroblasts. The selection of the PDGF signaling system seems to be cell-type dependent, since liver myofibroblasts showed predominant up-regulation of PDGF-β [37]. Considering that both the A chain and B chain of PDGF are not expressed in mesothelial cells, the autocrine action of PDGF is not expected to participate myofibroblastic conversion. Since PDGF was reported to be a very potent mitogenic factor of myofibroblast, the augmentation of PDGFR implies that it might serve as a proliferation trigger of mesothelial myofibroblasts [37]. This assumption does not conflict with our observation that TGF-β suppresses proliferation of cultured mesothelial cell (unpublished results), since previous study have showed that TGF-β in vitro may inhibit the proliferation of hepatic myofibroblasts, but promotes their proliferation in vivo by a PDGF autocrine loop [38]. The other factor that might be ascribed to cytokine system is secretogranin II (SCG2). SCG2 is the precursor of secretoneurin, a potent chemotactant of fibroblasts, monocytes, endothelial cells, and vascular smooth muscle cells [39].

The pathogenic nature of myofibroblastic conversion was implied by the potential risk of compromising cytoprotective capability against various cellular stresses or injuries (Table I, group G). One example was the down-regulation of heat shock 70 kD protein 8 (HSPA8, also termed Hsc70 or Hsp73) and heat shock 90 kD protein A (HSP90A) without concomitant elevation of other inducible heat shock proteins. HSPA8 is not only a con-

stitutively expressed molecular chaperone but also an adenosine triphosphatase (ATPase) that releases clathrin from coated vesicles [40]. We also observed suppression of some other constitutively expressed cytoprotective factors such as selenoprotein P (SEPP1), cytochrome P450 (CYP1B1), and aldehyde dehydrogenase (ALDH1A3) in myofibroblasts. These changes did not seem to be myofibroblast specific since TGF-β also induced similar suppression in liver cells [41, 42].

Finally, we observed alternations of some ubiquitously expressed cellular factors following myofibroblastic conversion (Table I, group H). The down-regulation of *Niemann-Pick disease, type C2* gene (NPC2) could cause a decrease of lysosomal protein called HE-1 (human epididymis-1) and affect cholesterol metabolism [43]. The decreased expression of heterogenous nuclear ribonucleoprotein U (HNRPU), small nuclear ribonucleoprotein polypeptide E (SNRPE), and Sam68-like phosphotyrosine protein (T-STAR) might affect RNA processing [44]. Other factors that might be suppressed include soluble protein-tyrosine phosphatase (PTPN11 or PTP2C), adipose differentiation-related protein (ADFP), and iron-binding protein, melanotransferrin (MFI2). The expression of α-mannosidase (MAN2B1), a lysosomal glycosyl hydrolase necessary for glycoprotein turnover, showed significant up-regulation.

The transdifferentiation of mesothelial cells to myofibroblasts after experimental TGF-β1 stimulation raises the possibility of therapeutic targeting of this response for prevention of fibrogenic peritoneal injury. The therapeutic strategies include blocking (*1*) initial proliferation of mesothelial epithelial cells; (*2*) transdifferentiation to myofibroblasts; and (*3*) proliferation of myofibroblasts. Recent studies have shown that ED-A domain of cellular fibronectin (cFN) might be a promising target that not only dramatically increases after TGF-β1 stimulation but also exhibits permissive activity of TGF-β–induced myofibroblastic transdifferentiation [3]. It is presumable a safe target due to its minimal expression in normal adult tissue [45].

It is a great challenge to make sense of the vast quantity of data generated by cDNA analysis of myofibroblastic conversion. Even though the cDNA array contains more than 3500 genes, this still does not represent a complete survey. We expect that we have identified only a fraction of the genes that are relevant to the myofibroblastic conversion. In addition, the biologic information extrapolated from these data is incomplete in a sense of lacking functional verification. Further functional study of these genes is essential for elucidating the pathogenic role of myofibroblastic conversion of mesothelial cells in peritoneal injury.

CONCLUSION

This study describes myofibroblastic conversion of mesothelial cells, a previously undefined, yet frequently speculated, cell adaptive or pathogenic process. Our study helps to elucidate the complex molecular and cellular events involved in this process. Our findings also suggest revoking the earlier concept of "multipotential subserosal cells" as progenitor cells of both epithelial mesothelial cells and myofibroblasts [46]. Our results indicate that differentiated mesothelial epithelial cells can convert or transdifferentiate to myofibroblasts, another differentiated status of mesothelial cells.

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